Human Reproduction, Vol.35, No.6, pp. 1325–1331, 2020
Advance Access Publication on May 12, 2020 doi:10.1093/humrep/deaa068

human reproduction

ORIGINAL ARTICLE ***Infertility***

Comparison of euploidy rates of blastocysts in women treated with progestins or GnRH antagonist to prevent the luteinizing hormone surge during ovarian stimulation

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Submitted on October 6, 2019; resubmitted on February 18, 2020; editorial decision on March 11, 2020

STUDY QUESTION: Does the prevalence of euploid blastocysts differ between patients treated with progestin primed ovarian stimulation (PPOS) and those treated with conventional ovarian stimulation?

SUMMARY ANSWER: The numbers of blastocysts and euploid blastocysts per patient and the number of euploid embryos per injected oocyte are similar for patients undergoing progestin-primed ovarian stimulation and for those undergoing conventional ovarian stimulation with GnRH antagonist.

WHAT IS KNOWN ALREADY: New approaches to ovarian stimulation have been developed based on the use of drugs administrable by mouth instead of via injections. Attention has been dedicated to progestins to block the LH surge. Previous data regarding the number of oocytes retrieved and the number of good-quality embryos generated in PPOS have demonstrated similar outcomes when compared to conventional ovarian stimulation, even if some concerns regarding the quality of embryos have been advanced.

STUDY DESIGN, SIZE, DURATION: This is a prospective non-inferiority age-matched case–control study. In a period of 6 months, a total of 785 blastocysts from 1867 injected oocytes obtained from 192 patients were available for analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Infertile women undergoing IVF and preimplanation genetic testing (PGT) cycles were included. Forty-eight patients were treated with PPOS, and for each of them three age-matched historical controls ($n = 144$) treated with a GnRH antagonist protocol were selected. PGT was performed according to next-generation sequencing technology.

MAIN RESULTS AND THE ROLE OF CHANCE: Basal characteristics were similar in the two groups; a substantial similarity of the main outcome measures in the two treatment groups has also been found. The rate of formation of euploid blastocysts per oocyte was 21% in both the two treatment groups. The percentage of patients with euploid embryos and the total number of euploid blastocysts per patient (median and interquartile range, IQR) in the PPOS group were 38.7 (25.5–52.9) and 2 (1.3–3.1), respectively. These figures were not significantly different in women treated with the GnRH antagonist protocol i.e. 42 (28–53.8) and 2.1 (1.3–2.9), respectively.

LIMITATIONS, REASONS FOR CAUTION: This was a case–control study which may limit the reliability of the main findings.

WIDER IMPLICATIONS OF THE FINDINGS: Our results encourage the use of PPOS, especially for oocyte donation, for fertility preservation and for patients in which total freezing of embryos is foreseen, for those expected to be high responders or candidates for preimplantation genetic testing. However, studies aiming to investigate the effect of PPOS on the live birth rate are warranted.

STUDY FUNDING/COMPETING INTEREST(S): None.

Key words: non-conventional ovarian stimulation / progestin primed ovarian stimulation / euploid blastocyst / euploidy rate / preimplantation genetic testing

Introduction

The growing application of IVF has made it preferable to employ techniques more convenient for the patients. Because of daily subcutaneous administration, the widespread utilization of GnRH analogues is burdened by important costs and poor manageability (the drug must be accurately prepared), aspects that potentially affect patients' adherence to the therapy. In recent years, GnRH antagonists have been largely preferred to GnRH agonists because of some advantages including reduced risk of cyst formation and ovarian hyperstimulation syndrome (OHSS). However, GnRH antagonists are expensive and sometimes difficult to manage with significant rates of cancellation and premature luteinizing hormone (LH) surge (Tarlatzis and Kolibianakis, 2007). An important medical alternative to inhibit spontaneous ovulation during ovarian stimulation is the use of oral progestins. A previous study by Letterie and colleagues had already demonstrated that a combination of ethinyl estradiol and norethindrone administered from Day 6 or 8 of the menstrual cycle for 5 days allowed folliculogenesis, but inhibited midcycle LH surge and, consequently, ovulation during controlled ovarian stimulation (Letterie, 2000). In agreement with this, different studies have demonstrated significant LH suppression by progestins during ovarian stimulation in the luteal phase (Kuang *et al*., 2014; Wang *et al*., 2016a), suggesting that the LH surge could have been transiently suppressed by high doses of progesterone. The essential assumption when using a progestin during ovarian stimulation is the total freezing of the entire cohort of embryos derived from retrieved oocytes, due to progesterone's known detrimental effect on the endometrium (Massin *et al*., 2017; La Marca *et al*., 2019).

The effectiveness and safety of progestins have been widely investigated in the recent years: the number of mature oocytes retrieved and the number of good-quality embryos generated have been shown to be similar in the protocols with the use of medroxyprogesterone acetate (MPA) when compared to the conventional ones (Kuang *et al*., 2015; Wang *et al*., 2016b; Sighinolfi *et al*., 2018; La Marca *et al*., 2019). No significant difference has been found between the two groups in the incidence of premature LH surge and in the experience of OHSS: moreover, in patients with polycystic ovary syndrome a possible reduction in the incidence of moderate to severe OHSS using MPA has been observed (Wang *et al*., 2016b). Similar results have been demonstrated also with the use of oral micronized progesterone (Zhu *et al*., 2015; Zhu *et al*., 2016; Zhu *et al*., 2017a; Wang *et al*., 2018) and dydrogesterone (Zhu *et al*., 2017b; Yu *et al*., 2018).

Nevertheless, some inconsistencies have been reported regarding reproductive outcomes with the use of MPA. In a recent randomized control trial in which PPOS has been compared to conventional stimulation in oocyte donors with the number of oocytes as primary outcome (Beguería *et al*., 2019), MPA was shown to be non-inferior to a GnRH antagonist in terms of number of mature oocytes retrieved at pick-up in oocyte donation cycles, but other reproductive outcomes were unexpectedly lower when it was used. By contrast, a very recent retrospective study showed similar implantation, clinical pregnancy and live birth rate in women treated with the two different stimulation strategies. Eighty-seven donors were treated with conventional ovarian stimulation in the first donation cycle and with PPOS in the second cycle. The use of PPOS was associated to a significant increase in the number of retrieved oocytes and to unmodified success rate when compared to the GnRH antagonist cycles (Yildiz *et al*., 2019).

Euploidy status is a very relevant marker of good embryo quality. As the number and rate of euploid blastocysts is directly related to the chance of having a live birth, an unmodified rate of euploidy in PPOS cycles may imply that the live birth rate would be non-inferior to what reported for conventional ovarian stimulation.

In this prospective non-inferiority, age-matched case–control study, we wished to investigate the euploid blastocyst formation rate in patients undergoing PGT-A cycles and treated with PPOS or conventional based ovarian stimulation.

Materials and Methods

Study subjects

This is a prospective case–control study of ART cycles performed at the European Hospital, Rome, Italy, between December 2018 and November 2019. PGT was proposed to infertile couples for reasons of advanced maternal age, recurrent miscarriage, repeated implantation failure or severe male infertility, as well as to all good-prognosis patients who desired information regarding the health status of their embryos. Patients were recruited without applying any particular exclusion criteria regarding baseline characteristics of patients such as age, main cause of infertility, ovarian reserve or body mass index (BMI). In total, data from 196 patients were available for the analysis: 48 patients recruited prospectively and who underwent progestin primed ovarian stimulation, and, for each of them, three historical controls patients matched for age who underwent conventional ovarian stimulation. Basal characteristics of patients are reported in Table I.

IVF/ICSI treatment protocol

Controlled ovarian stimulation started on the second day of the menstrual cycle, with an initial dose of FSH chosen according to age, antral follicle count or serum anti-Müllerian hormone (AMH) and BMI (La Marca and Sunkara, 2014; Grisendi and La Marca, 2017). In addition to the gonadotrophin, participants received either MPA (Farlutal, 10 mg, Italy) in the progestin primed ovarian stimulation or ganirelix (Orgalutran 0.25 mg/50 ml, MSD, Italy) in the conventional ovarian stimulation. MPA was administered orally in a single dose each day of the stimulation period until the day of the triptorelin acetate administration (trigger day). The prescribed dose has been used in previous publications to ensure pituitary inhibition (Kuang *et al*., 2015). Ganirelix was administered in the evening by subcutaneous daily injections (0.25 mg/day), from the seventh day of hormonal stimulation until trigger day. An ovarian ultrasound was performed on stimulation daya 5–6, and gonadotrophin doses were adjusted according to the ovarian response. When at least one follicle reached ≥18 mm, 0.3 mg of triptorelin acetate (Decapeptyl, Merck, Italy) was

Table I Demographic characteristics of the patients.

Variables	Conventional ovarian stimulation (COS)	Progestin primed ovarian stimulation (PPOS)
N	144	48
Female age (years)	37 (33–39)	37 (33–39)
BMI (kg/m^2)	22 (20.2–23.9)	22.3 (20–24.3)
Primary infertility (%)	77	72
Secondary infertility (%)	23	28
Female factor of infertility (%)	35	32
Male factor of infertility (%)	36	41
Combined factors of infertility (%)	29	27
Duration of infertility (months)	28 (18–41)	30 (19–39)
Current smokers (%)	19	19
Serum anti-Müllerian hormone (ng/ml)	3.4 (1.8–4.1)	3.2(1.9–3.9)
Serum Day 3 FSH (IU/L)	6.4 (5.8–7.8)	6.8 (5.5–7.8)
Antral follicle Count (*n*)	13.4 (9–23)	11.9 (9–25.5)

Unless stated; otherwise, values are median (quartiles)

administered to trigger ovulation; 36 h later, follicles were aspirated under patient sedation in both groups (oocyte pick-up, OPU).

Oocyte insemination, embryo culture and biopsy

All biologic procedures were performed as described elsewhere (Minasi *et al*., 2016). Briefly, after retrieval, cumulus–oocyte complexes were incubated in fertilization medium (Quinn Advantage Protein Plus Fertilization Medium; Sage) for 2–3 h at 37°C under the gas phase of 5% O_2 and 6% CO_2 (balance N_2) until denudation. For all oocytes, denudation was performed by exposure to 20 IU/ml of hyaluronidase fraction VIII (Hyaluronidase 80 U/ml in HEPES-HTF; Sage) in HEPES-buffered medium (Quinn Advantage Medium with HEPES; Sage). Subsequently, oocytes were aspirated in and out of a plastic pipette (Flexipet; 170- and 140-µm inner diameters; Cook) to allow the removal of cumulus and corona cells. Only oocytes with the first polar body extruded (metaphase II) were treated with the use of ICSI immediately after the denudation procedure. Finally, injected oocytes were moved to single drops of cleavage medium (Quinn Advantage Protein Plus Cleavage Medium; Sage) under oil at 37°C, 5% O_2, and 6% CO_2. On Day 3, for all of the developing embryos, a media change-over (Quinn Advantage Blastocyst Medium; Sage) for sequential culture was performed. On Day 3, a noncontact 1.48-µm diode laser (Fragouli *et al*., 2008) was used to create a circular 6–9-µm-diameter opening in the zona pellucida in cleavage-stage embryos to allow the trophectoderm cells to herniate. Depending on the embryo's development, the blastocyst stage can be reached on Day 5, 6, or 7. On the day of biopsy, 5–10 trophectoderm cells were gently aspirated into the biopsy pipette (Cook) followed by a laser-assisted removal from the rest of the blastocyst. The obtained trophectoderm cells were washed in sterile phosphate-buffered saline (PBS) solution and then placed in microcentrifuge tubes containing 2 µl PBS, spun down for a few seconds and sent to Genoma laboratory for analysis (Greco *et al*., 2014).

Preimplantation genetic analysis

For whole genome amplification (WGA), TE cell samples and negative controls were first lysed and genomic DNA was randomly fragmented and amplified using the SurePlex DNA Amplification System (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's protocol. This proprietary single tube technology is based on random fragmentation of genomic DNA and subsequent amplification by PCR utilizing flanking universal priming sites as previously described (Fiorentino *et al*., 2011).

Briefly, biopsies collected in 2.5 µl of 1× PBS were lysed using 2.5 µl of SurePlex cell extraction buffer and 5 µl of the SurePlex Extraction cocktail master mix and incubation at 75°C for 10 min followed by incubation at 95°C for 4 min. The random fragmentation of genomic DNA was done by adding 5 µl of SurePlex Pre-amplification cocktail to the lysed biopsy samples or to genomic DNA controls and incubating the mixture according to the following protocol: 1 cycle of 95°C for 2 min, followed by 12 cycles of 95°C for 15 s, 15°C for 50 s, 25°C for 40 s, 35°C for 30 s, 65°C for 40 s and 75°C for 40 s, followed by a hold at 4°C. Thereafter, 60 µl of freshly prepared SurePlex Amplification cocktail was added to the 15 µl of synthesis product in each reaction tube. Resulting mixtures were amplified according to the following thermal cycler programme: 1 cycle of 95°C for 2 min, followed by 14 cycles of 95°C for 15 s, 65°C for 1 min and 75°C for 1 min, followed by a hold at 4°C. To determine the success of the amplification, 5 µl of each amplified sample plus 5 µl gel loading buffer was examined by electrophoresis on a 1.5% agarose 1× TBE gel.

Libraries were prepared using the VeriSeq PGS workflow (Illumina, Inc.). DNA 'indexing' was performed using the VeriSeq Index Kit-PGS. During the library preparation step, the input DNA is tagmented (tagged and fragmented) by the Nextera™ XT transposome. The Nextera transposome simultaneously fragments the input dsDNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps. A limited-cycle PCR reaction uses these adapter sequences to amplify the insert DNA. The PCR reaction also adds index sequences on both ends of the DNA, thus enabling dual-indexed

sequencing. One nanogram of quantified dsDNA template at 0.2 ng/μl was added to 5 μl of Amplicon Tagmentation Mixture (ATM) and 10 μl of Tagmentation DNA Buffer (TD). The tagmented DNA was amplified via a limited-cycle PCR. PCR product clean-up used AM Pure XP beads (Beckman Coulter, Brea, CA, USA) to purify the library DNA. Purified libraries were eluted with 50 μl of the Nextera XT Resuspension Buffer. Each indexed library was normalized by beads and then multiplexed in 24-plex library pools.

Single-end, dual index 36 base pair reads (1 × 36 double index) sequencing was performed using the Illumina v3 chemistry workflow on a MiSeq sequencer with the MiSeq Reagent Kit v3-PGS (Illumina, Inc.), which contains the ready to load on-board clustering and SBS chemistry reagents. A sample sheet, used by both the MiSeq system and BlueFuse software, was generated using BlueFuse Workflow Manager. Reads were demultiplexed and aligned to the human genome hg19 by the on-instrument MiSeq Control Software (MCS v2.5). BAM files from the MiSeq system are imported directly into the BlueFuse Multi (4.3) analysis software (Illumina, Inc.) using the prepared sample sheet. BlueFuse Multi (v4.3) analysis software processes and displays the data to provide genomic profiles of each sample in a run. The samples acceptance criteria was a number of total reads >700 000 with a number of reads passing filter >500 000, and overall noise (DLR) ≤0.2.

Filtered reads from each sample were then mapped into the corresponding chromosome interval or bin. As previously described (Fiorentino *et al*., 2014), the count data in each bin was normalized using GC content, and *in silico* reference data in order to remove bias, and copy numbers were determined using of a combination of a Gaussian probability function (PDF; with copy number states 0–4 and a standard deviation of 0.33) and thresholding. The copy number state with the highest probability for a chromosome was used unless the distance to the next most probable copy number was >0.011. In that case, the median value of the most likely copy number states of all bins of a chromosome was used, set to a gain when >2.5 and to a loss when <1.5.

Outcomes and statistical analysis

The main outcome measure was the euploid blastocyst formation rate. With an expected euploid blastocyst rate per injected oocyte of 15% for a female population of a mean age of 38 (according to our own data), and aiming to exclude an absolute difference of 5% between the two strategies, a minimum of 330 oocytes per group should be injected to reach a power of 80% (alpha error 0.1, beta error 0.2). According to our own database, a mean of nine oocytes are retrieved per each single stimulation cycle; hence, at least 36 patients should be included in each group.

Continuous data were presented as median and inter-quartile range (IQR), while categorical data are presented as percentage. Parametric, nonparametric and Fisher's tests were used to compare baseline characteristics and outcomes as appropriate. All statistical tests used a two-tailed α of 0.05. Statistical analysis was done with the use of Stata 12 software. This study was approved by the local Ethical Committee.

Results

In total, 785 blastocysts obtained from 1867 injected mature oocytes were available for analysis. This sample size outnumbers the one required (see Materials and Methods section). Totally 48 women were treated with progestin primed ovarian stimulation and 144 with conventional ovarian stimulation. Demographic characteristics of the patients are reported in Table I. As expected, between the two groups there are no statistically significant differences in mean age, BMI, markers of ovarian reserve, duration of infertility and type of infertility.

The outcome of ovarian stimulation for IVF and PGT-A is reported in Tables II and III. No significant differences were found between the two groups. In particular, the total number of mature injected oocytes, 2PN and blastocysts, were similar in patients treated with the two different ovarian stimulation strategies. In women treated with PPOS, the rate of euploidy and the total number of euploid blastocysts were 38.7 (25.5–52.9) and 2 (1.3–3.1), respectively, and in women treated with the GnRH antagonist protocol, these figures were not significantly different: 42 (28–53.8) and 2.1 (1.3–2.9).

When considering results per injected oocyte (Table III), the blastulation rate and the rate of euploid blastocyst formation are the same in the two treatment groups (euploid blastocyst rate per oocyte: 21% in both the two groups of patients).

The rate of euploid embryos for each individual patient in the two treatment groups is reported in the Supplemental Fig. S1. There is a great overlapping of data between the two groups that indicates a similarity of the PGT-A outcome when the two treatment strategies were applied. Expectedly, there is no difference between the two regression lines that represent the correlation existing between women age and rate of euploid blastocysts in the two groups of patients.

Discussion

In our study, the rate of euploid formation per injected oocyte was similar in patients undergoing progestin-primed ovarian stimulation or conventional ovarian stimulation with GnRH antagonist control. As a consequence, the number of euploid blastocysts and embryo euploidy rate per patient are the same. This finding is relevant for clinicians, since some controversies have been raised about the impact of progestins on oocyte quality.

A dozen or so studies about PPOS are available in the literature so far, involving more than 2600 patients (see La Marca and Capuzzo (2019) for review). In the vast majority of retrospective and observational prospective studies, the PPOS has been demonstrated to be efficient and non-inferior to conventional stimulation when considering the number of retrieved oocytes, number of embryo and pregnancy rate. However, in a very recent study, reproductive outcomes were unexpectedly lower when MPA was used rather than a GnRH antagonist, with a statistically significant difference in biochemical pregnancy rate (44.3 versus 56.2%), clinical pregnancy rate (29.9% versus 42.5%) and live birth rate (27.4% versus 36.9%), even if the number of mature oocytes retrieved was similar between the two groups of patients in oocyte donation cycles (Beguería *et al*., 2019). It is worth mentioning that this trial was designed assuming the number of retrieved oocytes and not the pregnancy rate as primary outcome. A very recent retrospective study was in some way reassuring and in line with results from the present study as it reported a similar implantation rate of blastocysts from donors treated with PPOS and conventional ovarian stimulation when transferred to recipients. Expectedly the live birth rate was the same, as well (Yildiz *et al*., 2019).

Table II Characteristics and outcomes of ovarian stimulation.

Variables	Conventional ovarian stimulation (COS)	Progestin primed ovarian stimulation (PPOS)
N	144	48
Total FSH consumption (IU)	2630 (2050–3100)	2750 (2100–3000)
Duration of stimulation (days)	11 (10.5–11.5)	11.8 (11–12.5)
Triggering with hCG (%)	35	35
Triggering with GnRH (%)	65	65
Estradiol level on triggering day (ng/ml)	2850 (2300–3650)	2430 (2150–3450)
Number of COCs (*n*)	14.3 (9–18)	14 (8–19)
Number of MII (*n*)	9.5 (5.5–12.3)	9.5 (5.8–13)
Number of 2PN (*n*)	7.2 (4.1–9.6)	7.4 (4.5–11.2)
Total number of blastocysts	590	195
Blastocysts per patient (*n*)	4.1 (2.1–5.8)	4 (2–5.5)
Number of euploid blastocysts per patient (*n*)	2 (1.3–3.1)	2.1 (1.3–2.9)
Euploidy rate per patient (%)	42 (28–53.8)	38.7 (25.5–52.9)

Data are median (quartiles). COC: cumulus oocyte complex; MII: mature oocytes; 2 PN: 2 pronuclear.

Table III Numbers of oocytes and blastocysts resulting from ovarian stimulation.

Variables	Conventional ovarian stimulation (COS)	Progestin primed ovarian stimulation (PPOS)
Total number of retrieved oocytes, *n*	1944	672
Total number of injected MII oocytes, *n*	1411	456
Number of 2PN, *n*	1037	355
Number of Blastocysts, *n*	590	195
Euploid blastocysts, *n*	302	96
Euploid blastocysts per injected MII, %	21%	21%

COC: cumulus oocyte complex; MII: mature oocytes; 2 PN: 2 pronuclear.

Results from our study are consistent with this last study: PPOS may have no impact on the embryo quality, at least when assessed by analysing the chromosomes of the embryos. If confirmed in future studies, these findings may lead to the acceptance of more comfortable and user-friendly stimulation strategies, as progestins are administered orally, and to a reduction in costs for ovarian stimulation which may in turn increase patients' access to IVF treatments. Since oocytes and embryos freezing and delayed transfer are mandatory, PPOS has already been proposed as the best option in women undergoing fertility preservation and oocyte donation, since these conditions do not require consequent embryo transfer (Massin, 2017). Moreover, the use of progestins to inhibit ovulation could be particularly beneficial for patients at risk of OHSS: since ovulation in these women is often co-triggered by hCG and a GnRH agonist, a lower dose of hCG is required, minimizing the risk of early-onset OHSS. As well, freezing all the embryos with delayed transfer can reduce by a large extent the risk of late onset OHSS.

Orally administrable, progestins have also been shown to be extremely beneficial in economic terms compared to GnRH analogues, and easier to administer. The application of progestins to inhibit ovulation in ovarian stimulation has been therefore shown to be effective and safe, with good results in terms of number and quality of the oocytes and embryos obtained and low OHSS risk. In particular, our study demonstrates that total number of blastocysts, number of euploid blastocysts and embryo euploidy are similar when PPOS and conventional stimulation are used. Further studies are needed on long-term obstetrical outcomes before this protocol can be introduced large-scale.

The approach with progestins permits an efficient control of pituitary LH secretion, lower costs and an easier access and administration for women by oral assumption. For this, PPOS could be the first choice for ovarian stimulation in fertility preservation. Moreover, PPOS could be proposed as a first-choice treatment in all cases in which ovarian stimulation and oocyte retrieval are not followed by the fresh embryo transfer. Ovarian stimulation in oocyte donors and in PGT-A and PGT-M cycles may be obviously based on progestins instead of GnRH analogue administration. As well as all the so-called 'non conventional' protocols for ovarian stimulation (luteal and random start, double ovarian stimulation) implying the 'freeze all' and segmentation of the cycle may be associated to the use of PPOS. Other patients that can benefit from progesterone block protocols are those at risk of OHSS. One relevant advantage of the association between a progestin and gonadotropins in high responders is that the triggering may be exerted by the GnRH agonist, and this helps to avoid early-onset

OHSS. As well, cryopreservation of all embryos with delayed transfer can diminish the risk of late onset OHSS.

Limitations of our study include the study design, which is a case–control study and the relatively small number of patients included. Only 48 women were treated with PPOS. However, data on euploidy have been obtained from a total of 785 embryos which may be considered a sufficiently high number to be confident with the main conclusions. In any case, we recognize that the study would have been more informative if data related to the implantation rate of all the embryos obtained would have been available.

In conclusion, in our study we demonstrated that PPOS is not associated to any detrimental effect on embryo quality, and assuming a similar implantation rate of euploid blastocysts from all patients, an unmodified live birth and cumulative live birth is expected to be found for PPOS when compared to conventional ovarian stimulation strategies.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

A.L.M. designed the study, performed statistical analysis, wrote the manuscript. M.C. performed statistical analysis, wrote the manuscript. S.S. managed the database. M.G.I. drawn tables and figures. F.S. collected the data and contributed to the interpretation of the results. M.T.V. collected the data and contributed to the interpretation of the results.M.G.M. collected the data, contributed to the interpretation of the results, wrote the manuscript. P.G. collected the data and contributed to the interpretation of the results. F.F. collected the data and contributed to the interpretation of the results. E.G. collected the data and contributed to the interpretation of the results, wrote the manuscript.

Funding

No specific funds were used for this article.

Conflict of interest

None.

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